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(71) Applicants *(for all designated States except US)*: **IMPERIAL COLLEGE INNOVATIONS LIMITED** [GB/GB]; Sherfield Building, Imperial College, London SW7 2AZ (GB). **TECHNION-ISRAEL INSTITUTE OF TECHNOLOGY** [IL/IL]; Technion City, Haifa 32000 (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NEMIROVSKY, Yael** [IL/IL]; Technion-Israel Institute of Technology, Dept. of Electrical Engineering, Technion City, Haifa 32000 (IL). **CHAYEN, Naomi, Esther** [GB/GB]; Biological Structure and Function Section, Division of Biomedical Sciences, Sir Alexander Fleming Building, Imperial College School of Medicine, London SW7 2AZ (GB).

(74) Agent: **MILES, John, S.**; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

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[Continued on next page]

(54) Title: NUCLEATION-INDUCING MATERIAL

a

b

(57) Abstract: The present invention relates to a method of crystallising a macromolecule comprising the step of adding a porous material to a crystallisation sample wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm, and provides materials, kits and systems useful in said method.

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Nucleation-inducing material

The present invention relates to the use of a novel nucleant in the crystallisation of macromolecules, and methods of crystallising proteins using the novel nucleant.

To date, nucleation of crystals has been facilitated mainly by seeding, epitaxy, charged surfaces or mechanical means (Stura, E.A. In *Protein crystallization: techniques, strategies, and tips.* (ed Bergfors, T.M.) (International University Line, La Jolla; 1999); McPherson, A. & Shlichta, P. *Science* **239**, 385-387 (1988); Sanjoh, A. & Tsukihara, T. *J. Cryst. Growth* **196**, 691-702 (1999); Visuri, K. *et al. Bio/Technology* **8**, 547-549 (1990)). Nucleation of crystals the necessary first step in the crystallisation process, which influences it decisively. Consequently, the ability to control it is of primary importance in crystallisation experiments. Nucleation presents a free energy barrier which must be overcome in a specific way, different from the supersaturation conditions which subsequently make crystal growth an energetically favourable process (Feher, G. & Kam, Z. *Methods Enzymol.* **114**, 77-112 (1985)). Formation of nuclei in the bulk of a solution is a stochastic process where protein molecules interact until a critical size aggregate is formed. Any environment that favours a higher local concentration of macromolecules provides a potential nucleation point and may lower the energy barrier for nucleation.

Pilot structural genomics projects show the success rate of getting from clone to structure to be about 10%. Production of crystals suitable for X-ray crystallography is found to be the rate-limiting step (e.g. the Human Proteome Structural Genomics pilot project; Brookhaven National Laboratory, The Rockefeller University and Albert Einstein College of

Medicine: http://proteome.bnl.gov/progress.html). It is currently necessary to find methods that will help to overcome this stumbling block.

The ultimate aim would be to find a 'universal' nucleant, which would promote crystallisation of a very wide range of proteins under a very wide range of conditions. Previous studies attempting to find nucleants have been undertaken by introducing candidate substances into crystallisation trials in a controlled manner (McPherson and Schlichta *Science* **239**, 385-387 (1988); Chayen, N.E., Radcliffe, J.W. & Blow, D.M. *Protein Sci.* **2**, 113-118 (1993); Blow, D.M., Chayen, N.E., Lloyd, L.F. & Saridakis, E. *Protein Sci.* **3**, 1638-1643 (1994)). Some have been useful for individual proteins but none have yet turned out to be of general use. Various other attempts to induce nucleation on irregular or rough surfaces, or surfaces of special composition (poly-L-lysine, plastic) have also proved generally ineffective (Chayen, N.E. & Saridakis, E. *J. Cryst. Growth* (in press)).

Sakamoto *et al. Nature* **408**, 449-453 (2000) and Dusastre *Nature* **408**:417 (2000) describe methods of characterising mesoporous materials and report the structures of some mesoporous materials. They are suggested to be useful in various areas of chemistry such as catalysis and molecular filtration but their use in crystal nucleation is not suggested.

We have found that porous materials with particular pore characteristics are useful as crystallisation nucleants. The materials are considered to have pores which may entrap macromolecules, for example protein molecules, and encourage them to nucleate and form crystals.

A first aspect of the invention provides a method of facilitating the crystallisation of a macromolecule comprising the step of adding a porous

material to a crystallisation sample wherein the porous material comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane and wherein the pore dimensions within the material have a variability of at least 10nm.

By use of the term "porous material" we mean a material which contains pores or cavities whose minimum dimension in any plane is at least 2nm and whose maximum dimension in any plane is less than 200nm. The pores may be interconnected so that the contents of one pore or cavity are accessible to one or more adjacent or connected pores or cavities. Preferably the pores or cavities are interconnected. However, it is preferred if the dimensions of adjacent or connected pores are not taken into account when determining the dimensions of any one pore. Preferably the pores of the porous material useful in the present invention are at least 2nm in the smallest dimension, and more preferably between 5nm and 10nm. Natural minerals have previously been used to promote nucleation (McPherson and Schlichta *Science* **239**, 385-387 (1988)) with some success. Although some of them were effective for some proteins, they were not effective in as many cases as we show in Example 1.

It is preferred that the average dimension of the pores in any plane is no larger than 200nm, 150nm or 100nm, more preferably no larger than 50nm, 30nm or 20nm. Still more preferably, the dimension of the pores is no larger than 10nm on average, in any plane. Preferably, the average dimension of the pores in any plane is between 5nm and 10nm.

Typically, the "porous material" which is useful in the present invention is distinguished by the non-uniform distribution of the pore sizes within it. Hence, it is preferred if the pores in the porous material are not uniform in

size, and have a high variability in the pore size. Hence, the material may have a proportion of pores which are uniform in size and do not differ significantly from each other in their dimensions in any plane, and a proportion of pores which differ significantly from each other in their dimensions in any plane. By "uniform" we mean that a dimension in any plane does not vary by more than +/-1nm, or +/- 2nm, and the dimension is shared by at least 5%, 10%, 20%, 30%, 40%, 50%, 60% or 70% of the pores in the material.

Of the population of pores which are not uniform in size, it is preferred if the minimum and/or maximum dimension in any plane of those pores varies from each other by at least 3nm, 5nm, 10nm,, more preferably at least 15nm, 20nm, 30nm, 40nm or 50nm variability, and still more preferably at least 60nm, 70nm, 80nm, 90nm or 100nm. It is further preferred if the pore dimensions of the non-uniform proportion of the pores vary between pores by at least 110nm or 120nm . Preferably, the variation in maximum and/or minimum pore dimensions between non-uniform pores is no greater than 200nm, more preferably by less than 175nm or 150nm.

Hence, one pore (which is not a pore of "uniform size") in a material may have a minimum dimension in any plane of 10nm, and any other pore in the same material (which also is not a pore of "uniform size" as described above) may have a minimum dimension in any plane of 50nm. This would provide a variability of 40nm in the minimum dimension between pores in that proportion of pores which are variable. Preferably, the variability is in the minimum pore dimension.

Preferably, the population of pores which vary as described above represents at least 10% of the pores, more preferably 20%, 30%, 40% or

50%, still more preferably 60%, 70% or 80% of the pores in the material. More preferably, at least 90% or 95% or 100% of the pores are variable in size.

Preferably the variation is within the range where the minimum dimension in any plane is 5nm and the maximum dimension is 20nm (ie has a distribution range of 5-20nm).

Preferably, in the porous material useful in the invention at least 20%, 30%, 40%, more preferably 50%, 60%, 70%, 80% or 90% of the pores has a dimension falling within the range of 5nm to 20nm.

In a preferred embodiment, the material is porous silicon and has a pore dimension distribution range of 5 to 20nm in a population of pores as defined above. In other words, at least 20%, 30%, 40%, more preferably 50%, 60%, 70%, 80% or 90% of the pores has a dimension falling within the range of 5nm to 20nm.

In a further preferred embodiment, the material is mesoporous glass and has a pore dimension distribution range of 5 to 20nm in a population of pores as defined above. In other words, at least 20%, 30%, 40%, more preferably 50%, 60%, 70%, 80% or 90% of the pores has a dimension falling within the range of 5nm to 20nm.

By the term 'mesoporous glass' we also encompass porous ceramics, for example of the type discussed in Fibbri *et al* (1995) *Biomaterials* vol. 16, 225-228.

It will be appreciated that a convenient average pore size for a particular application may depend on the size of the macromolecule to be crystallised. For example, larger macromolecular assemblies (ie with a size larger than 5nm or 10nm or 15nm Stokes' radius) such as virus capsids etc. may require bigger pores (ie, pores with an average size larger than 10nm or 20nm or 50nm in any one plane) than the pore size required by smaller macromolecular assemblies, such as those with a Stokes' radius of 5nm or less.

Figure 1 shows an electron micrograph of the structure of a porous material useful in the present invention, porous silicon. The structural properties of the porous silicon include a skeleton of the porous silicon layer which preserves the crystalline structure and direction of the silicon wafer. In this case, the pores have a columnar structure. However, porous silicon is crystalline and retains its crystallinity. Pores sizes in the porous silicon are controlled by the electrochemical process (current density). However, there is always a distribution in the pores' dimensions (here most are in the range 5 – 20 nm). Pore sizes can be determined by Scanning Electron Microscopy and porosity by weighing. The combination of the porosity and the activity of the silicon surface due to its electronic structure may play a role in providing the effect useful in the present invention.

Materials such as Sephadex™ beads and alumina powders have been tried previously in the crystallisation of macromolecules, and were found to be ineffective as general-use nucleants (Chayen, N.E. & Saridakis, E. *J. Cryst. Growth* (in press)). Sephadex™ comprises a meshwork produced by cross-linking of the substituent material. This meshwork effectively creates pores, the size of which are determined by the degree or type of cross-linking. It will be appreciated that Sephadex™ and sand are not included in

the present definition of "porous material". A difference between the porous material useful in the present invention and Sephadex™ is that that the pores in each given type of Sephadex bead are of quasi-uniform size. As described above, the porous material useful in the present invention comprises pores of variable sizes as defined above; the material has an average pore diameter generated by a fairly wide Gaussian-type spread of pore size. Hence the spread of pore dimension in the porous material is wider than that of cross-linked dextrans. Where the porous material useful in the present invention is porous silicon such as an etched crystalline silicon, another difference between the porous material useful in the present invention and Sephadex™ is the crystalline structure of the silicon substrate and its electrostatic properties.

It will be understood that where the term "macromolecule" is used, we include any molecule over 1kDa. Preferably the macromolecule is a biological macromolecule such as a nucleic acid, and more preferably the macromolecule is a polypeptide. Preferably the polypeptide comprises at least 10, 20 or 50 amino acids, more preferably at least 75, 100, 200, 500 or 1000 amino acids.

In a preferred embodiment of this aspect of the invention, crystallisation of the macromolecule is induced at a lower critical level of super saturation than that obtained where the porous material is not added to the sample.

In a further or alternative preferred embodiment, the conditions of the crystallisation sample is one which comprises conditions of supersaturation that are favourable to the crystal growth, but are inadequate or insufficient for spontaneous nucleation. Such conditions are a means to maximise the chances of obtaining crystals during initial screening of crystallisation

conditions, and provide a means of growing crystals at metastable conditions, at which the slower growth and the lack of excess and secondary nucleation often provide for growth of larger, better diffracting crystals. Determination of which supersaturation level is inadequate or insufficient for spontaneous nucleation is well known in the art of crystallisation. The level of supersaturation can be determined by setting-up screens covering a range of conditions around the conditions that yield crystals/microcrystals spontaneously.

According to a preferred embodiment, the porous material is an engineered porous material. By "engineered" we mean that the material is not one which exists in nature. Preferably, the porous material is porous silicon. Porous silicon is a crystalline silicon specially treated by electrochemical etching. A suitable porous silicon may be obtained from Technion Institute, Haifa, Israel. The silicon wafers useful in the preparation of such a suitable porous silicon are provided by Siltronics, Germany, and the method of production of a suitable porous silicon is described in detail in Example 1.

In a further preferred embodiment the porous material is mesoporous glass. Mesoporous glasses are described, for example, in Saravanapavan and Hench (2001) *J Biomed Mater Res* 54, 608-618; Fibbri *et al* (1995) *Biomaterials* vol. 16, 225-228 and may be particularly suitable in relation to the present invention.

Where the porous material is porous silicon as described above, the silicon may have any oxidation state which is chemically feasible for such silicon. Preferably the silicon has no oxidation or minimal oxidation. Silicon is instantaneously oxidized upon exposure to ambient atmosphere and a native oxide is formed approximately 10-20 Å thick, which increases in thickness with time in the case of porous silicon. Oxidation of the porous silicon can be avoided by storing the porous silicon in methanol or ethanol solution. Oxidation should be avoided since the oxide layer may fill the pores; hence, where pores in the porous silicon are 5-10nm, preferably the oxide layer is not more than about 2-3nm in thickness. By "minimal" oxidation we mean less than about 5% to about 20% oxidised. Oxidation can be observed by a colour change; unoxidised silicon is black, whereas oxidised silicon is ginger in colour.

A second aspect of the invention provides a method of facilitating the crystallisation of a macromolecule comprising the step of adding porous silicon material or mesoporous glass material to a crystallisation sample.

Porous silicon is described above, and a suitable porous silicon is available from Technion Institute, Haifa, Israel. Preferably, the porous silicon is one as described in Example 1.

A third aspect of the invention provides a method of preparing a porous material which comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm for use as a nucleant in crystallisation comprising cleaving said material into pieces of sub-millimetre dimensions.

Suitable and preferred porous materials are as described above.

A fourth aspect of the invention provides a method of preparing a porous silicon or mesoporous glass for use as a nucleant in crystallisation comprising cleaving said material into pieces of sub-millimetre dimension.

In a preferred embodiment, the material is porous silicon or mesoporous glass as described above, and preferably has a pore dimension distribution range of 5 to 20nm in a population of pores as defined above. In other words, at least 20%, 30%, 40%, more preferably 50%, 60%, 70%, 80% or 90% of the pores has a dimension falling within the range of 5nm to 20nm.

The porous material or porous silicon of the third and fourth aspects of the invention may be prepared *de novo* in pieces of sub-millimetre dimensions. This can be achieved by making the porous silicon on small micron size areas of silicon wafer. Part of the silicon wafer can be masked by masking material, for example, photoresist, so that only small micron sized bare silicon windows or areas are exposed to electrolyte solution. Several micron sized regions of porous silicon can be made on silicon wafer in this way. The wafer can then be cut if necessary into several small pieces, according to the area of porous silicon. Where the material has not been so prepared, it may be reduced in size to be in pieces of sub-millimetre

dimensions. Preferably, the porous material or porous silicon is converted into pieces or fragments which are no more than 200μm, 150μm or 100 micron in any dimension, more preferably no more than 75μm or 50μm, and still more preferably no more than 25μm in any dimension. More preferably the pieces are no more than 10 micron in any dimension. Advantageously, the pieces of porous material or porous silicon resemble a fine dust. Conversion of a porous material or porous silicon into fragments may be achieved by using a diamond cutter or by etching, such as chemical etching.

By "cleavage" we mean that the starting material is rendered into smaller fragments or pieces. Cleavage may be by any suitable technique, including cutting with a scalpel or by mechanical means (such as using a diamond cutter) or by breaking smaller pieces off the larger one using tweezers. Typically, the porous material useful in the present invention cannot be broken by hand in small enough pieces. The surface is mechanically fragile, so grinding or crushing is less preferred. It will be appreciated that to be useful as a nucleant in crystallisation, the fragments or pieces should be such that the network of pores within the material is exposed. In other words, the pores should be accessible by suitably sized molecules (ie, those which are not bigger than the size of the pores) when contacted by said molecules. Hence, fragments or pieces of the porous material are not sealed externally in any way by the cleavage process.

The cleavage method may be manual (as described above), or may be mechanical or one which employs a motorised machine.

A fifth aspect of the invention provides a method of determining the structure of a macromolecule comprising the steps of (i) crystallising the

macromolecule in the presence of a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm; and (ii) analysing the crystal structure of the crystal produced in step (i).

The porous materials which are useful and/or preferred in the fifth aspect of the invention are those as defined in more detail above. Preferably, the porous material is produced by the method of the third or fourth aspect of the invention. The material may be combined with the crystallisation sample components in any order. Preferably, the material is added before nucleation or growth of the crystallisation has started.

In a preferred embodiment, the porous material is porous silicon.

In a further preferred embodiment the porous material is mesoporous glass.

Nucleation may be detected by any suitable means; either directly, for example by using a microscope, or indirectly, for example by determining the light scatter characteristics as described in Rosenberger *et al J. Cryst. Growth* 129:1-12 (1993).

Methods of analysing the crystal structure of a crystal are well known in the art and are described in detail in (Drenth, J. *Principles of protein X-ray crystallography*. Springer-Verlag, New-York, 1994).

A sixth aspect of the invention provides a use of a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or

comprises pores with a minimum dimension of at least 2nm and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm in the crystallisation of a macromolecule.

Preferred macromolecules and suitable and preferred porous materials are defined above.

Porous materials which comprise pores with a minimum dimension of at least 2nm and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm, especially engineered porous materials, have not previously been contemplated as nucleants in macromolecule crystallisation. Their usefulness in nucleation may be due to the ability of the macromolecules to be crystallised to encounter a pore of a suitable size, be retained by the pores, and as a result be joined by other similar molecules. The retention of the macromolecules in a pore forms a local concentration suitable for nucleation.

A seventh aspect of the invention provides a kit of parts comprising a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm and a crystallisation agent.

Preferably, the porous material is in fragments of various sizes in sealed boxes containing ethanol. Preferably the kit further comprises extra ethanol and a cutting device.

Suitable and preferred porous materials are as defined above.

By "crystallisation agent" we include any one or more of a range of precipitants such as polymers and organic solvents and crystallisation agents such as salts. Specific examples of suitable precipitants include polyethylene glycol 400, polyethylene glycol 4000, mono-sodium dihydrogen phosphate and ammonium sulphate.

It will be appreciated that the kits of the invention are suitable for most crystallisation methods, including the microbatch, vapour diffusion hanging drop, sitting drop and sandwich drop crystallisation methods.

The kits of the invention may further comprise crystallisation plates or slides and/or filters. Where the kit further comprises crystallisation plates, it is preferred if the plates are multi-well plates.

In a preferred embodiment of this aspect, the kit further comprises oil for layering over the crystallisation sample. Suitable oils include paraffin such as that available from Hampton Research, CA 92677-3913 USA, catalogue number HR3-411.

In an alternative embodiment, the kit may be one which is suitable for screening for the crystallisation of macromolecules. In this embodiment, the kit may further include any one or more of a range of buffers (which may cover a range of pH values) and/or any one or more of a range of salts. Suitable buffers and salts are known in the art of crystallisation and include Na-HEPES pH 7.5, Tris hydrochloride pH 8.5 as buffers, and 0.2M ammonium sulphate and 0.2M sodium acetate trihydrate as salts.

Advantageously, the kit may further comprise multi-well crystallisation plates and filters.

An eighth aspect of the invention provides an automated method of crystallising a macromolecule comprising adding a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a distribution range of at least 10nm to a crystallisation trial using an automated dispensing system.

Suitable and preferred porous materials are as described above. It is particularly preferred if the porous material is a porous silicon or mesoporous glass as defined above.

Advantageously, the crystallisation is part of a screen or optimisation for crystallisation conditions.

The porous material may be prepared for administration to the trial by any suitable means. Preferably, the material is in the form of fine fragments, of no more than 10 micron in any dimension. In a preferred embodiment, the porous material is prepared according to the second or third aspect of the invention.

It will be appreciated that the porous material may be added as a single grain or particle or piece, or it may be added as a suspension in a liquid. Ensuring that only a single particle which may be only 10 micron in size is dispensed into an automated trial may be awkward, or may involve an unacceptably high level of error, such that no particle, or too many particles

are added. Clearly then, an advantage of the method where the porous material is added as a suspension is the ability to manipulate the material more easily, especially automatically using an automated liquid dispensing system. Forming a suspension of the material particles in a liquid such that dispensing a particular volume of the liquid is likely to include at least 1 piece or fragment of the material, but not too many pieces or fragments, would facilitate the step of adding the fragment to a crystallisation sample.

Hence, in a preferred embodiment of this aspect of the invention, the porous material is dispensed by an automated liquid dispensing system as a suspension.

Automated liquid dispensing systems are known in the art of protein crystallisation, and an example is the IMPAX system produced by Douglas Instruments, Hungerford, UK. In this system, several components of the crystallisation sample can be dispensed from separate reservoirs through the same tip into a single sample drop. Suitably, the automated system useful in this aspect of the invention is programmable such that defined volumes and concentrations of liquid or suspension may be dispensed into the crystallisation drop.

The crystallisation trial may be any suitable method, including microbatch and vapour diffusion. It is preferred if the automated method is microbatch technique (Chayen *et al* (1990) *Appl. Cryst.* **23**:297; Chayen *et al* (1992) *J. Crystal Growth* **122**:176). The method may be modified as described in D'Arcy *et al* (1996) *J. Crystal Growth* **168**:175-180 to use a mixture of silicon and paraffin oil over the crystallisation sample. To maintain the level of supersaturation which is suited to crystal growth, and which does not promote nucleation, it is preferred if the oil used is one which does not

permit detectable diffusion of water molecules to the oil-air interface, and therefore one which does not promote a concentration of the components of the crystallisation sample. Hence it is preferred if the oil is a branched paraffin in the C20+ range, and not a silicon fluid (such as a polymer of dimethylsiloxane units).

A ninth aspect of the invention provides a crystal obtainable or obtained by the method of the first, second or eighth aspects of the invention.

As described above, it is likely that use of the porous material in the crystallisation material causes the macromolecule to be crystallised to nucleate within the pore network of the material, and not on the surface of the porous material. Consequently, the resulting crystal may include the porous material within the actual crystal structure of the crystal produced. Such crystals may be distinct from crystals obtained by alternative means (such as using a mineral surface to promote nucleation), since the nucleant may form an integral part of the crystal, and not a peripheral part.

A further aspect of the invention provides a use for an automated liquid dispensing system in the method of the eighth aspect of the invention, wherein the porous material to be dispensed is in the form of a suspension.

A "automated liquid dispensing system" includes any suitable automated dispensing system capable of dispensing a volume of a suspension of the porous material which is between 0.1µl and 1ml. Preferably the system is one as described above.

The invention will now be described in more detail with the aid of the following Figures and Example:

Figure 1

A cross-section of porous silicon showing the structure of the material (tree like); the silicon is white while the dark areas are pores in the layer.

Figure 2

A working phase diagram determined using microbatch for catalase at 18°C, showing in particular the supersolubility curve. The variables are concentrations of protein and PEG 6K. The other conditions are as in the Table. The two arrows show the conditions (metastable) at which porous silicon induced nucleation of large crystals in drops which otherwise remained clear.

Figure 3

Crystals growing on and/or in the proximity of porous silicon fragments.

a. Lysozyme. Area shown is 2.5 x 1.8 mm
b. Trypsin. Area shown is 2.2 x 2.0 mm.
c. Phycobiliprotein. Area shown is 3.0 x 2.3 mm.
d. Phycobiliprotein close-up showing a crystal stuck onto a silicon fragment. Area shown is 1.1 x 1.0 mm.
e. Same crystal still stuck after having moved the fragment. Area shown is 1.0 x 0.8mm.

Example 1

Use of porous silicon as a crystallisation nucleant.

Microporous and mesoporous silicon materials consist of networks of pores and cages, electrochemically etched on a crystalline silicon surface. Porous materials have been highlighted recently in Nature (Sakamoto *et al*, (2000)

Nature **408**, 449-453), concerning their uses in various areas of chemistry, chemical engineering, semiconductor research and the development of physical, chemical and biological sensors. Use of such materials in macromolecular crystal nucleation has previously never been considered.

We tested the suitability of specially made porous silicon as a nucleant for crystallisation, and found that the material functioned as a nucleant with various model and a target proteins

A thin layer of porous silicon of 15 μm was electrochemically fabricated on a silicon substrate using a backside aluminium contact. The silicon substrate was lightly boron-doped p-type single side polished, so that the average pore size was 5-10 nm. The dimensions of the pores exhibit a Gaussian distribution, with an estimated standard deviation of 3 nm. A picture of a cross section of the porous silicon material can be seen in Figure 1, exhibiting a cleaved silicon substrate with a thin porous silicon layer on top of it. The resulting thin wafers of material were easily cut into pieces of sub-millimetre dimensions and were immersed in crystallisation solutions. Experiments were performed using the two major crystallisation methods, namely vapour diffusion hanging drops and microbatch drops dispensed under a layer of oil (Chayen (1997) *Structure* 5:1269-1274).

Nucleation requires very specific, restricted conditions. In order to find these conditions, we experimentally determined a "working phase diagram" for each protein, an example of which, for catalase, is shown in Figure 2. Such a phase diagram shows the supersolubility curve, which is the threshold above which the protein molecules spontaneously aggregate (as crystals or amorphous precipitation). The porous silicon was inserted at

conditions just below the supersolubility curve (shown by arrows in Figure 2).

The effect of porous silicon was tested on a variety of proteins: catalase, concanavalin A, lysozyme, a phycobiliprotein, thaumatin and trypsin, of Stokes' radii ranging from 2 to 5 nm. The crystallisation trials were also chosen to reflect a selection of different common precipitating agents and a wide range of pH.

Porous silicon was successful in inducing nucleation at conditions where the solution otherwise remained clear (metastable), leading to the growth of large single crystals of diffracting quality in 5 of the 6 proteins tested (Table 1 and Figure 3). In some cases, crystals grew only on the silicon fragment, leaving the rest of the drop completely clear (Figure 3a). In other cases, one or more crystals were also growing in other parts of the drop, their numbers and sizes decreasing with distance from the main silicon fragment (Figure 3b, c). It is possible that some nuclei diffused away from the nucleation site after their formation, or that nucleation also took place on extremely small pieces of the material which had remained loosely attached to the main fragment after cleavage. However, the possibility of other mechanisms of facilitation of nucleation not localised on the nucleant, due for example to the creation of protein concentration gradients, cannot be excluded. In order to ensure that the crystals were actually attached to the fragments when they were seen to be so, and not just lying above or below them, we turned the fragments in the solutions using microtools. In all cases, the crystals remained attached to them (e.g. Figure 3d, e). The porous silicon was not effective in the case of concanavalin A. Other porous materials, e.g. (alumino)silicates of uniform pore sizes up to 5nm (VPI-5 and MCM-41[12]) were also investigated in the course of this study but were not found to

influence the nucleation process.

Porous silicon induces nucleation of crystals of a fairly wide range of proteins, at conditions of supersaturation that are favourable to the crystal growth of each, but are inadequate or insufficient for spontaneous nucleation. The discovery of such agents has three-fold importance. Firstly, they can provide a means to maximise the chances of obtaining crystals during initial screening of crystallisation conditions (particularly useful in the structural genomics era). Secondly, they can be used to grow crystals at metastable conditions, at which the slower growth and the lack of excess and secondary nucleation often provide for growth of larger, better diffracting crystals. Thirdly, they represent a first step towards the 'universal' nucleant or nucleants.

Experimental

A porous silicon layer was formed on a boron-doped p-type crystalline silicon bulk with an aluminium backside contact, using an electrochemical cell with HF:Ethanol (1:1) solution, rinsing in ethanol. (The HF aqueous solution had a concentration of 48% v/v). The 15 μm thick layers with 65 % porosity were prepared over 10 min with a current density of 30 mA/cm^2. In the cell, water molecules oxidise the silicon during the electrochemical anodisation process. The SiO_2 layer is subsequently etched by the HF, and the ethanol is used only to increase the ability of the solution to penetrate and keep all the volume wet during the electrochemical process. Very small H_2 bubbles could be seen while the current was flowing through the cell. This is expected since the oxygen of the water molecules is the oxidising agent while the hydrogen is released. The samples were stored in ethanol prior to use as a nucleation substrate. In this way, the formation of a native

silicon oxide was significantly reduced.

The main structural information about the pores is that there is a columnar structure. However, porous silicon is crystalline and retains its crystallinity. Pores sizes are controlled by the electrochemical process (current density). However, there is always a distribution in the pores' dimensions (here most are in the range 5 – 20 nm). We determined pore sizes by Scanning Electron Microscopy and porosity by weighing.

Bovine liver catalase (Cat. no. C-9322), jack bean concanavalin A type IV (C-2010), hen egg-white lysozyme (L-6876), thaumatin from Thaumatococcus daniellii (T-7638) and porcine pancreas trypsin (T-0134) were purchased from Sigma (Steinheim, Germany). The phycobiliprotein was prepared and purified in-house. Polyethylene glycol of mean molecular weight 6,000 (PEG 6K), 2-Methyl-2,4-pentanediol (MPD) and the various salts used were also purchased from Sigma.

The supersolubility curves were established using the IMPAX automated crystallisation system (Chayen, N.E., Shaw-Stewart, P.D. & Blow, D.M. *J. Cryst. Growth* **122**, 176-180 (1992)). This was done by screening around published conditions for these proteins, except in the case of the phycobiliprotein, which had not been previously crystallised. The published conditions are as follows: Catalase: Hampton Research Catalogue, Vol.5, no.2, 1995, p.23. Others: BMCD Database: http://www.bmcd.nist.gov:8080/bmcd/bmcd.html. Crystal codes: lysozyme: C06E; thaumatin: C26A; trypsin: C1CC.

In order to control nucleation, it is necessary to work in very clean conditions. Hence, the lysozyme, trypsin and thaumatin stock solutions

were filtered with a 300,000 M.W. cut-off filter, and catalase and the phycobiliprotein with a 0.22μm mesh size filter (Ultrafree-MC, Millipore, Bedford, USA) before setting up the experiments. Concanavalin A was not filtered because part of the protein appeared to be sticking to the filter. The porous silicon coated wafers were broken into small pieces (ca. 0.06 mm^2) and placed inside the droplets set at various conditions below the supersolubility curves of the proteins. Both microbatch and hanging-drop vapour diffusion set-ups were used, with drop volumes ranging from 2 to 5 μl.

For the microbatch trials, Terazaki-type plates were purchased from Nunc (Denmark). The fragments of porous silicon wafer were placed on the bottom of the plates' wells (depressions); the crystallisation drops were dispensed onto the fragments in the wells and covered with paraffin oil. For the vapour diffusion trials, Linbro-type crystallisation plates contained 1 ml of the reservoir solutions. The drops and the silicon fragments were dispensed on silanised glass coverslips that were inverted above the wells and sealed with Apiezon C oil (M&I, Manchester, UK). All the experiments were run at 18°C.

The porous silicon is etched on the surface of a crystalline silicon wafer, which is embedded on an aluminium support. It is thus an integral part of the silicon wafer.

Protein	Molec. Weight (kDa)	Metastable conditions	Protein conc. (mg/ml)
Lysozyme	14.5	6%(sat) sodium chloride 20 mM sodium citrate, pH 4.6	36

Trypsin	24	30-32%(sat) ammonium sulphate 100 mM Tris, pH 8.4	12-20
Catalase	232	5.1-5.4%(w/v) PEG 6K, 5%(v/v) MPD 100 mM Tris, pH 7.5	11.5-12.5
Thaumatin	22	0.34 M sodium-potassium tartrate 50 mM PIPES, pH 6.8	16
Phycobiliprotein	126	0.6-0.7 M ammonium sulphate 40 mM MES, pH 6.1 1.5 mM dodecyl maltoside	11

Table 1: Conditions under which the proteins crystallised in the presence of porous silicon (no crystals grow in these conditions in the absence of porous silicon).

CLAIMS

1. A method of facilitating the crystallisation of a macromolecule comprising the step of adding a porous material to a crystallisation sample wherein the porous material comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane and wherein the pore dimensions within the material have a variability of at least 10nm.

2. A method of facilitating the crystallisation of a macromolecule comprising the step of adding a porous silicon and/or mesoporous glass to a crystallisation sample.

3. A method according to Claim 1 or 2 wherein crystallisation of the macromolecule is induced at a lower critical level of super saturation than that obtained where the porous material is not added to the sample.

4. A method of preparing a porous material which comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane and wherein the pore dimensions within the material have a variability of at least 10nm for use as a nucleant in crystallisation comprising cleaving said material into pieces of sub-millimetre dimensions.

5. A method of preparing a porous silicon and/or mesoporous glass for use as a nucleant in crystallisation comprising cleaving said material into pieces of sub-millimetre dimensions.

6. A method according to Claim 4 or 5 wherein the pieces are no more than 200 micron in any dimension

7. A method according to Claim 6 wherein the pieces are no more than 100 micron in any dimension.

8. A method according to Claim 4 to 7 wherein the cleavage is by cutting with a scalpel or mechanical means (diamond cutter) or breaking smaller pieces off a larger one using tweezers.

9. A method of determining the structure of a macromolecule comprising the steps of:

(i) crystallising the macromolecule in the presence of a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm; and

(ii) analysing the crystal structure of the crystal produced in step (i).

10. Use of a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm in the crystallisation of a macromolecule.

11. A kit of parts comprising a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a

minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm and a crystallisation agent.

12. A kit according to Claim 11 wherein the porous material is in fragments of various sizes in sealed boxes containing ethanol.

13. A kit according to Claim 11 or 12 which further comprises extra ethanol and a cutting device.

14. An automated method of crystallising a macromolecule comprising adding a porous material wherein the porous material is porous silicon and/or mesoporous glass and/or comprises pores with a minimum dimension of at least 2nm in any plane and a maximum dimension of less than 200nm in any plane wherein the pore dimensions within the material have a variability of at least 10nm to a crystallisation trial using an automated dispensing system.

15. A method according to Claim 14 wherein the crystallisation is in a screen or optimisation.

16. A method according to Claim 14 or 15 wherein the porous material is added as a suspension in a liquid.

17. A method according to Claim 1 to 3, 9 or 14 to 16 or a use according to Claim 10 or a kit according to any one of Claims 11 to 13 wherein the porous material or porous silicon and/or mesoporous glass is prepared according to the method of any one of Claims 4 to 8.

18. A method according to any one of Claims 1 to 9 or 14-17 or a use according to Claim 10 or a kit of parts according to any one of Claims Claim 11 to 13 wherein the porous material is porous silicon and/or mesoporous glass.

19. A method or use or kit according to Claim 18 wherein the silicon has a minimal oxidation.

20. A method, use or kit according to Claim 18 or 19 wherein at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the pores of the porous silicon have a dimension falling within the range of 5nm to 20nm.

21. A method, use or kit according to Claim 20 wherein the porous silicon and/or mesoporous glass has an average pore size of 5-10nm.

22. A crystal obtainable or obtained by the method of any one of Claims 1 to 3 or 14-21.

23. A method according to Claim 1 to 3 or 9 or 14-21 or a use according to Claim 10 or 16-21 or a crystal according to Claim 22 wherein the macromolecule is a biological macromolecule.

24. A method or use according to Claim 23 wherein the macromolecule is a protein.

25. Use of an automated liquid dispensing system to dispense a porous material or porous silicon and/or mesoporous glass according to the method of Claim 16.

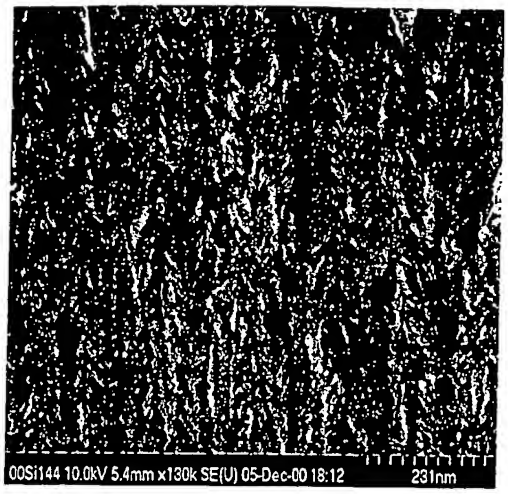
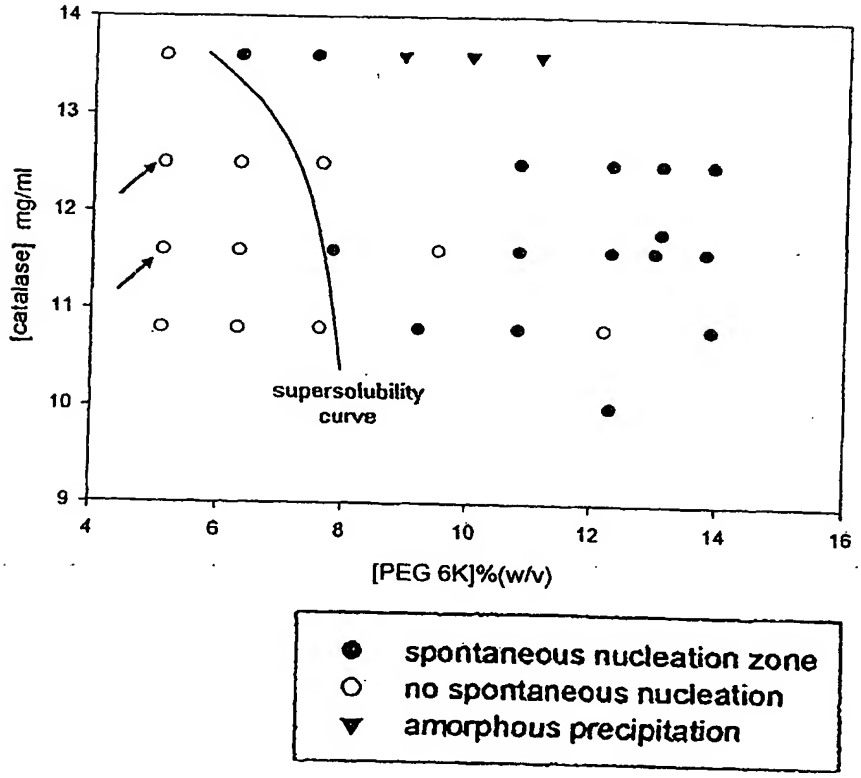


Figure 1

Figure 2


14
13
12
11
10
9
[catalase] mg/ml
4
6
8
10
12
14
16
[PEG 6K]%(w/v)
supersolubility curve
spontaneous nucleation zone
no spontaneous nucleation
amorphous precipitation

a

b

Figure 3: page 1

c

d

e

Figure 3: page 2

INTERNATIONAL SEARCH REPORT

itional Application No
PCT/GB 02/01921

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C30B29/58 C30B7/00 G01N23/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C30B G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, CHEM ABS Data, MEDLINE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 909 841 A (SUMITOMO METAL IND) 21 April 1999 (1999-04-21) abstract page 16, column 2, line 31 - line 42	1-15, 17-20, 22-24
X	US 6 171 512 B1 (SATO NOBUHIKO ET AL) 9 January 2001 (2001-01-09) abstract column 4, line 11 - line 17	4-7, 18-20
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2002	21/08/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Celler, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/01921

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAKAMOTO ET AL: "Direct imaging of the pores and cages of three-dimentional mesoporous materials" NATURE, vol. 408, 23 November 2000 (2000-11-23), pages 449-453, XP002206071 cited in the application page 451, column 1, paragraph 4; figure 3 page 452, column 1, last paragraph	4-7, 18-21
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P,X	US 6 277 662 B1 (NAGATA SEIICHI) 21 August 2001 (2001-08-21) abstract column 1	4-7, 18-21
P,X	CHAYEN ET AL: "Porous silicon: an effective nucleation-inducing material for protein crystalisation" J MOL BIOL, vol. 312, 28 September 2001 (2001-09-28), pages 591-595, XP002206075 the whole document	1-25

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/GB 02/01921

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 22-24 (partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 22-24 (partly)

Present claim 22, and the dependent claims 23,24, relate to an extremely large number of possible products. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the protein crystals disclosed in Table 1, on pages 23 and 24 of the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procédure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/01921

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